miRNA Microarray System with miRNA Complete Labeling and Hyb Kit

Protocol

For use with Agilent miRNA microarrays

Version 2.4, September 2011

Microarrays manufactured with Agilent SurePrint Technology

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Agilent Technologies

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Manual Part Number

G4170-90011

Edition

Version 2.4, September 2011

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Rd
Santa Clara, CA 95051 USA

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In this Guide...

This document describes Agilent's recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent oligonucleotide microarrays for microarray-based miRNA systems.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment, as well as total RNA extraction recommendations) that you should read and understand before you start an experiment.

2 Procedures

This chapter describes the steps to prepare Spike-In solutions (optional), samples, hybridize, wash and scan miRNA microarrays, and to extract data using the Feature Extraction Software.

3 Characterization of Total RNA

This chapter contains instructions for characterization of total RNA.

4 Reference

This chapter contains reference information related to the protocol and the limited use license.

What's New in 2.4

- Support for Agilent SureScan Microarray Scanner.
- Update to list of recommended RNA extraction methods.

What's New in 2.3

- Introduction of SurePrint G3 8x60K microarray format, which allows more content to be assayed on a single array without any changes to sample processing. The Sureprint G3 8x60K format requires the use of the Agilent C scanner.
- Requirement to use Feature Extraction 10.7.3 with the use of the microRNA Spike-In kit.

What's New in 2.2

- Optional column purification during sample preparation.
- Optional use of microRNA Spike-In Kit.
- Requirement to use Feature Extraction 10.7 with the use of the microRNA Spike-In kit.

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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE Agilent cannot guarantee microarray performance and does not provide technical support to those who use non-Agilent protocols in processing Agilent's microarrays.

Procedural Notes

- Determine the integrity of the input RNA for labeling and hybridization prior to use to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

WARNING

- **Dimethyl Sulfoxide (DMSO) may be harmful by inhalation, ingestion or skin absorption. DMSO vapor or mist is irritating to the eyes, mucous membranes and upper respiratory tract. It can cause skin irritation and allergic respiratory and skin reactions. Overexposure may cause reproductive disorder(s) based on tests with laboratory animals.**
- **2X Hi-RPM Hybridization Buffer is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). It may be harmful if swallowed. The buffer contains material that may cause damage to the skin and central nervous system. Please refer to the MSDS for complete safety information.**

Agilent Oligo Microarray Kit Contents

- Eight microarrays printed on a 1-inch × 3-inch glass slide; one or three slides per kit.
- CD containing microarray design files in various file formats.

For more information on microarray designs visit the following Web site: http://www.chem.agilent.com

To get design files or create a custom design for any species in the miRBASE, go to the Agilent eArray Web site at http://earray.chem.agilent.com.

NOTE

Store entire kit at room temperature. After breaking foil on microarray pouch, store microarray slides at room temperature (in the dark) under a vacuum dessicator or N_2 purge box. Do not store microarray slides in open air after breaking foil.

Required Equipment

Table 1 Required equipment

Description	Company and part no.
Microarray Scanner	Agilent p/n G4900DA, G2565CA or G2565BA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides 8 microarrays/slide, 5 slides/box	Agilent p/n G2534-60014
Hybridization oven 20 rpm capability; temperature set at 55°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Nuclease-free 1.5 mL microcentrifuge tubes	Eppendorf p/n 022363204
Magnetic stir bar (×2)	Corning p/n 401435 or equivalent
Magnetic stir plate	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	
Microcentrifuge	Eppendorf p/n 5417R or equivalent
Slide-staining dish, with slide rack (×3)	Thermo Shandon p/n 121 or equivalent

Table 1 Required equipment (continued)

Description (continued)	Company and part no.
Circulating water baths or heat blocks set to 16°C, 37°C, and 100°C.	Corning p/n 6795-420 or equivalent
Vacuum concentrator with heater	Savant SpeedVac p/n SPD111V or equivalent
1.5L glass dish	Pyrex p/n 7211 or equivalent
Clean forceps	
Ice bucket	
Pipetman micropipettors, (P-10, P-20, P-200, P-1000) or equivalent	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Vortex mixer	
Timer	
N_2 purge box for slide storage	

Required Reagents

Table 2

Description	Company and part no.
miRNA Complete Labeling and Hyb Kit	Agilent p/n 5190-0456
Gene Expression Wash Buffer Kit	Agilent p/n 5188-5327
Milli-Q water or equivalent	

Optional Equipment/Reagents

Table 3

Description	Company and part no.
MicroRNA Spike-In Kit	Agilent p/n 5190-1934
Universal miRNA Reference Kit	Agilent p/n 750700
2100 Bioanalyzer	Agilent p/n G2938A
RNA 6000 Nano Kit	Agilent p/n 5067-1511
RNA 6000 Pico Kit	Agilent p/n 5067-1513
Ozone Barrier Slide Covers	Agilent p/n G2505-60550
Small RNA Kit	Agilent p/n 5067-1548
MicroBioSpin 6 Columns	Bio-Rad p/n 732-6221
Slide box	Corning p/n 07201629
PCR 96 plate	Eppendorf p/n 951020401
Dye filter	Sigma p/n Z361569
Thermocycler	

Required Hardware and Software

Table 4

Description
Agilent G4450AA Feature Extraction software 9.5 or later without Spike-In or Feature Extraction 10.7.3 with Spike-In
Agilent Scan Control software, version A. 7.0 or later (includes XDR functionality)
Pentium IV 1.5 GHz or higher
512 MB RAM (1 GB recommended)
20 GB available disk space (if saving images and results files locally)

Table 4

Description
Microsoft Windows 2000 with SP2 or later (fully tested on SP4), Windows XP SP2
Microsoft Internet Explorer 5.5 or later
Adobe Acrobat Reader 4.0 or later
Agilent GeneSpring software GX10.0 or later (optional)

RNA Extraction Recommendation

Total RNA extraction methods differ in numerous ways and may impact:

- yield
- the inclusion of small RNAs in the total RNA extraction
- the quantification of the total RNA

The Agilent miRNA Microarray system is for use with total RNA extracts. Do not use size fractionation or small RNA enrichment protocols. The following method has been validated on the Agilent miRNA Microarray system:

- Absolutely RNA miRNA Kit - Agilent p/n 400814

To get consistent results for comparative experiments, use the same total RNA extraction method. Different total RNA extraction methods can result in slightly different miRNA profiles.

CAUTION

Do not use purified miRNA. This protocol supports the use of total RNA only.

Extraction methods that use organic solvents such as TRIZOL can result in inaccurate quantification, because organic solvent contamination from carry-over during the RNA extraction can compress the 260/230 ratio. The affected 260 measurement can result in inaccurate quantification of the total RNA. To ensure the removal of residual TRIZOL reagent, do one to two additional chloroform extractions after the final extraction step in the TRIZOL protocol, before you continue to the ethanol precipitation. See "Characterization of Total RNA" on page 49 for more information on RNA purity and quality assessment.

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Agilent's miRNA Microarray System uses cyanine 3-labeled targets to measure miRNA in experimental and control samples. Figure 1 is a standard workflow for sample preparation and array hybridization.

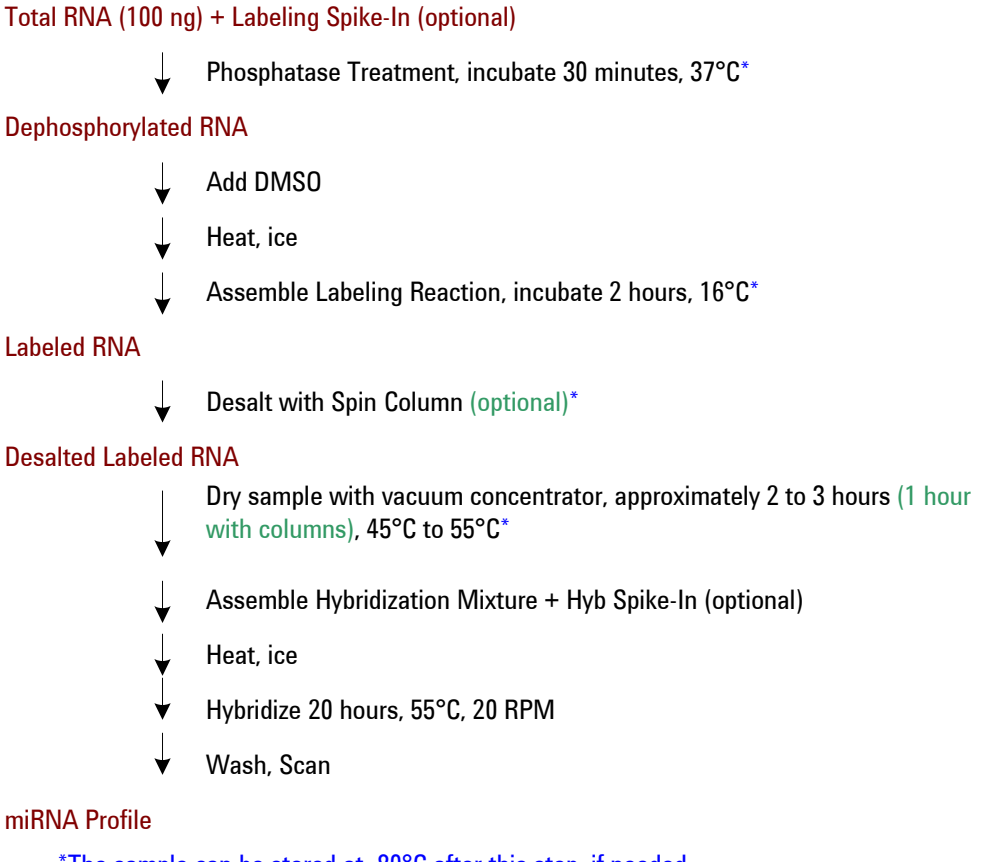
Total RNA (100 ng) + Labeling Spike-In (optional)
Phosphatase Treatment, incubate 30 minutes, 37°C*
Dephosphorylated RNA
Add DMSO
Heat, ice
Assemble Labeling Reaction, incubate 2 hours, 16°C*
Labeled RNA
Desalt with Spin Column (optional)*
Desalted Labeled RNA
Dry sample with vacuum concentrator, approximately 2 to 3 hours (1 hour with columns), 45°C to 55°C*
Assemble Hybridization Mixture + Hyb Spike-In (optional)
Heat, ice
Hybridize 20 hours, 55°C, 20 RPM
Wash, Scan
miRNA Profile
*The sample can be stored at -80°C after this step, if needed.

Figure 1 Workflow for sample preparation and array processing with the optional Bio-Rad columns and spike-ins.

Spike-In Solution Preparation

Agilent's microRNA Spike-In kit (p/n 5190-1934) consists of two microRNA Spike-In solutions for process control. Use the Spike-In solutions to help distinguish significant biological data from processing issues. The Labeling Spike-In solution is to be spiked into the labeling reaction while the Hyb Spike-In solution is to be spiked into the hybridization reaction.

To handle and store Spike-In solutions

CAUTION

Do not use spike-in solutions for normalization purposes.

Do not use with drosophila miRNA microarrays. Spike-in solutions are derived from drosophila sequences.

1. Thaw all components.
2. Briefly spin tubes.
3. Label 5 tubes “1st Dilution Labeling Spike-In” and an additional 5 tubes “1st Dilution Hyb Spike-In”.
4. Add 198 µL of the Dilution Buffer to each of the 10 tubes.
5. Add Spike-In solution:
   - Add 2 µL of the Labeling Spike-In solution to each of the 5 tubes that are labeled “1st Dilution Labeling Spike-In”.
   - Add 2 µL of the Hyb Spike-In solution to each of the 5 tubes that are labeled “1st Dilution Hyb Spike-In” tubes.
6. Mix well and briefly spin tubes.
7. (*Optional*) To increase the number of uses of this kit, and to prevent excess freeze/thaw cycles, store aliquots of the spike-in solutions in tubes that are appropriately labeled “1st Dilution Labeling Spike-In” and “1st Dilution Hyb Spike-In”. Store at -80°C.
8. Use only one aliquot of the “1st Dilution Labeling Spike-In” and “1st Dilution Hyb Spike-In” at a time and store the other aliquots at -80°C.

To prepare Spike-In solutions

NOTE Prepare dilutions of the Spike-In solution just prior to use.

1. Label 4 new tubes:
   - 2nd Dilution Labeling Spike-In
   - 3rd Dilution Labeling Spike-In
   - 2nd Dilution Hyb Spike-In
   - 3rd Dilution Hyb Spike-In
2. Into each of the 4 tubes, add 198 µL of nuclease-free water.
3. Make the 2nd dilution solutions:
   - Add 2 µL from the 1st Dilution Labeling Spike-In tube into the 2nd Dilution Labeling Spike-In tube.
   - Add 2 µL from the 1st Dilution Hyb Spike-In tube into the 2nd Dilution Hyb Spike-In tube.
4. Mix well and spin briefly in a centrifuge.
5. Make the 3rd dilution solutions:
   - Add 2 µL from the 2nd Dilution Labeling Spike-In tube into the 3rd Dilution Labeling Spike-In tube.
   - Add 2 µL from the 2nd Dilution Hyb Spike-In tube into the 3rd Dilution Hyb Spike-In tube.
6. Mix well and briefly spin in a centrifuge.

   This dilution is used in the labeling reaction or the hybridization reaction.
7. Discard the 2nd Dilution and 3rd Dilution after use.
8. Store 1st Dilution tubes at -80°C.

CAUTION Do *not* freeze/thaw the original or 1st Dilution Spike-In solution more than twice.

Sample Labeling and Hybridization

Agilent's miRNA Complete Labeling and Hyb Kit (p/n 5190-0456) generates fluorescently-labeled miRNA with a sample input of 100 ng of total RNA. This method involves the ligation of one Cyanine 3-pCp molecule to the 3' end of a RNA molecule with greater than 90% efficiency. The Agilent miRNA Complete Labeling and Hyb Kit provides all the needed components for sample labeling and hybridization preparation. These include:

- Calf Intestinal Alkaline Phosphatase (CIP)
- 10X Calf Intestinal Phosphatase Buffer
- T4 RNA Ligase
- 10X T4 RNA Ligase Buffer
- Dimethyl Sulfoxide (DMSO)
- Nuclease-Free Water
- Cyanine 3-pCp
- 10X GE Blocking Agent
- 2X Hi-RPM Hybridization Buffer

NOTE

Please refer to "Characterization of Total RNA" on page 49 for procedural recommendations on total RNA characterization.

Read the protocol in its entirety before you continue.

Step 1. Prepare the labeling reaction

This protocol is compatible with hybridization of labeled miRNA to either 8x15K or 8x60K microarray formats.

Dephosphorylation

1 Dilute total RNA sample to 50 ng/µL in 1X TE pH 7.5 or DNase/RNase-free water.

2 Add 2 µL (100 ng) of the diluted total RNA to a 1.5 mL microcentrifuge tube and maintain on ice.

3 Immediately prior to use, prepare the Calf Intestinal Alkaline Phosphatase (CIP) Master Mix from:

- Table 5 when using the Labeling Spike-In solution (3rd Dilution)
- Table 6 when not using the Labeling Spike-In solution

Add the components in the order indicated and maintain on ice.

Table 5 CIP Master Mix with Labeling Spike-In solution

Components	Volume (µL) per reaction	Volume (µL) per 9 reactions
10X Calf Intestinal Phosphatase Buffer	0.4	3.6
Labeling Spike-In	1.1	9.9
Calf Intestinal Phosphatase	0.5	4.5
Total Volume	2.0	18.0

Table 6 CIP Master Mix without Labeling Spike-In solution

Components	Volume (µL) per reaction	Volume (µL) per 9 reactions
10X Calf Intestinal Phosphatase Buffer	0.4	3.6
Nuclease-Free Water	1.1	9.9
Calf Intestinal Phosphatase	0.5	4.5
Total Volume	2.0	18.0

4 Add 2 µL of the CIP Master Mix to each sample tube for a total reaction volume of 4 µL. Gently mix by pipetting.

5 Dephosphorylate the sample by incubating the reaction at 37°C in a circulating water bath or heat block for 30 minutes.

If needed, the samples can be stored at -80°C after incubation.

NOTE A thermocycler, in conjunction with PCR plates, can be used to process a large number of samples. Use a PCR cap to seal the plates. Do not use tape.

Denaturation

1 Add 2.8 µL of 100% DMSO to each sample.

2 Incubate samples at 100°C in a circulating water bath or heat block for 5 to 10 minutes.

CAUTION Incubate the sample for no less than 5 minutes and no more than 10 minutes, or the labeling efficiency of the sample will be affected.

3 Immediately transfer to ice-water bath.

CAUTION You must use an ice-water bath and not just crushed ice to ensure that the samples remain properly denatured.

When using a thermocycler, transfer the PCR plate to an ice-water bath immediately after incubating at 100°C to prevent the RNA from re-annealing. Thermocyclers do not cool as quickly as is needed.

4 Continue to the next step immediately.

Ligation

1 Warm the 10X T4 RNA Ligase Buffer at 37°C and vortex until all precipitate is dissolved.

CAUTION Make sure that the 10X T4 RNA Ligase Buffer has cooled to room temperature before you proceed. Failure to do so will affect the T4 RNA Ligase activity, and thus the labeling efficiency.

CAUTION The Cyanine3-pCp is light-sensitive. Please minimize any exposure to light.

2 Immediately prior to use, prepare the Ligation Master Mix by gently mixing the components listed in Table 7 and maintain on ice.

Table 7 Ligation Master Mix for T4 RNA Ligase

Components	Volume (µL) per reaction	Volume (µL) per 9 reactions
10X T4 RNA Ligase Buffer	1.0	9.0
Cyanine3-pCp	3.0	27.0
T4 RNA Ligase	0.5	4.5
Total Volume	4.5	40.5

CAUTION Be sure to use the Ligation Master Mix within 15 minutes of mixing all the components in Table 7. Failure to do so may affect the labeling efficiency.

3 Immediately add 4.5 µL of the Ligation Master Mix to each sample tube for a total reaction volume of 11.3 µL.

4 Gently mix by pipetting and gently spin down.

5 Incubate at 16°C in a circulating water bath or cool block for 2 hours.

Step 2. Purify the labeled RNA (optional)

If you are not using the columns for purification, continue at "Step 3. Dry the sample" on page 24.

This step removes DMSO and free Cyanine3-pCp. The use of the column reduces the needed drying time in "Step 3. Dry the sample", but it adds hands-on processing time.

Micro Bio-Spin 6 column preparation

1. Invert the column sharply several times to resuspend the settled gel and to remove any air bubbles.
2. Snap off the tip and place into a 2 mL microcentrifuge tube supplied with the columns from Bio-Rad.
3. Remove the green cap from the column. If the buffer does not drip into the 2 mL microcentrifuge tube, press the green cap back onto the column and remove it again. Let the buffer drain for about 2 minutes.
4. Check to make sure that all columns are evenly drained.
5. Discard the drained buffer from the 2 mL microcentrifuge tube and then place the column back into the tube.
6. Spin the microcentrifuge tube containing the column for 2 minutes at 1000 x g in a centrifuge.

CAUTION The speed of the microcentrifuge must be accurately set (for example, 1000 x g, *not* 1000 rpm). If the spin speed is too low, sample can be lost. If the spin speed is too high, desalting may be ineffective and the column may break down.

7. Remove the column from the 2 mL microcentrifuge tube and place it into a clean 1.5 mL microcentrifuge tube. Discard the 2 mL microcentrifuge tube.

Sample purification

1. Add 38.7 µL of RNase-Free Water or 1X TE pH 7.5 to the labeled sample for a total volume of 50 µL.
2. Without disturbing the gel bed, pipette the 50 µL sample onto the gel bed from step 7 above.
3. Elute the purified sample by spinning the microcentrifuge tubes containing the columns for 4 minutes at 1000 x g in a centrifuge.
4. Discard the columns and keep the miRNA sample-containing flow-through on ice.
5. Check that the final flow-through is translucent and slightly pink. The flow-through volume needs to be uniform across the samples and close to 50 µL.

CAUTION Do not discard the final flow-trough. It now contains the labeled miRNA sample.

Step 3. Dry the sample

1. After the 16°C labeling reaction or sample purification, completely dry the samples. Use a vacuum concentrator at 45 to 55°C or on the medium-high heat setting. This step may take up to 1 hour after column purification and up to 3 hours without column purification.
2. Check the samples after 30 minutes. Continue vacuum concentration until they are dry.

   To check for sample dryness, flick hard on the tube and make sure that the pellets do not move or spread.

CAUTION The sample must be completely dried after labeling. Residual DMSO will adversely affect the hybridization results.

Step 4. Prepare the 10X Blocking Agent

1. Add 125 µL of nuclease-free water to the vial containing lyophilized 10X GE Blocking Agent supplied with the Agilent miRNA Complete Labeling and Hyb Kit (p/n 5190-0456).
2. Mix by gently vortexing. If the pellet does not go into solution completely, heat the mix for 4 to 5 minutes at 37°C.
3. Drive down any material adhering to the tube walls or cap by centrifuging for 5 to 10 seconds at 17,000 x g.

NOTE 10X Blocking Agent can be prepared in advance and stored at -20°C for up to 2 months. After thawing, repeat the vortexing and centrifugation procedures before use.

Step 5. Prepare hybridization samples

1. Equilibrate water bath or heat block to 100°C.
2. Resuspend the dried sample in 17 µL of nuclease-free water when the Spike-In solution is used and 18 µL when the Spike-In solution is not used.
3. *(Optional)* Add 1.0 µL of the Hyb Spike-In solution (3rd Dilution) to each sample.
4. Add 4.5 µL of the 10X GE Blocking Agent to each sample.
5. Add 22.5 µL of 2X Hi-RPM Hybridization Buffer to each sample for a total of 45 µL.
6. Mix well but gently on a vortex mixer.

Table 8 Hybridization mix for miRNA microarrays with Hyb Spike-In solution

Components	Volume (µL)
Labeled miRNA sample	17
Hyb Spike-In	1
10X GE Blocking Agent	4.5
2X Hi-RPM Hybridization Buffer	22.5
Total Volume	45

Table 9 Hybridization mix for miRNA microarrays without Hyb Spike-In solution

Components	Volume (µL)
Labeled miRNA sample	18
10X GE Blocking Agent	4.5
2X Hi-RPM Hybridization Buffer	22.5
Total Volume	45

For best results, stagger the tubes in sets of eight such that eight arrays on one array slide can be loaded at once. Have the SureHyb chamber along with the gaskets and microarrays easily available to prepare the hybridization assembly before you continue to the next step.

7 Incubate at 100°C for 5 minutes.

8 Immediately transfer to an ice water bath for 5 minutes.

CAUTION Do not leave samples in the ice water bath for more than 15 minutes. Longer incubations may adversely affect the hybridization results.

9 Quickly spin in a centrifuge to collect any condensation at the bottom of the tube.

10 Immediately proceed to "Step 6. Prepare the hybridization assembly" on page 28.

Step 6. Prepare the hybridization assembly

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (G2534-90002) for in-depth instructions on how to load slides, assembly and disassembly of chambers, as well as other helpful tips. This user guide is available with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

Go to http://www.genomics.agilent.com, and search on **Running a Microarray Experiment** to view an instructional video.

To process the miRNA system microarray:

1 Load a clean gasket slide into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.

For Agilent scanning and Feature Extraction:

- Position the slides so that the barcode label is to your left.
- Load the samples left to right. For 8-pack slides, start with the first row. The output files will come out in that same order. Refer to "Array/Sample tracking on an 8-pack array slide" on page 59 for guidelines on tracking sample position for multipack slide formats.

NOTE If you are not processing all of the arrays on a slide, place unused gasket wells at the far end opposite of the barcode. In the unused wells, pipette 45 µL of 1X hybridization buffer.

2 Slowly dispense all of the volume of the hybridization sample onto the gasket well in the center.

Heating and cooling the hybridization sample results in varying degrees of volume loss. To adjust for the variation in final sample volume, adjust your pipette to avoid the introduction of air bubbles during application to the gasket well. To avoid leakage from the gasket well, minimize the introduction of bubbles during sample loading.

CAUTION Do not allow the pipette tip or the hybridization solution to touch the gasket walls. Allowing liquid to touch the gasket wall greatly increases the likelihood of gasket leakage.

3 Slowly place an array "active side" down onto the SureHyb gasket slide, so that the "Agilent"-labeled barcode is facing down and the numeric barcode is facing up. Verify that the sandwich-pair is properly aligned.

CAUTION When you assemble the array slide to the gasket slide, keep the array slide parallel to the gasket slide at all times, and do not drop the array slide onto the gasket slide. Doing so increases the chances of samples mixing between gasket wells.

4 Place the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.

5 Firmly hand-tighten the clamp onto the chamber.

6 Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. If necessary, tap the assembly on a hard surface to move stationary bubbles.

7 Place assembled slide chamber in rotisserie in a hybridization oven set to 55°C. Set your hybridization rotator to rotate at 20 rpm.

8 Hybridize at 55°C for 20 hours.

CAUTION Be sure that the arrays are hybridized for at least 20 hours. Hybridization can occur for longer than 20 hours but the actual hybridization time should be consistent if the results are to be compared. Failure to maintain consistent hybridization times may adversely affect your data.

CAUTION If you are not loading all the available positions on the hybridization rotator rack, be sure to balance the loaded hybridization chambers on the rack so that there are an equal number of empty positions on each of the four rows on the hybridization rack.

NOTE The **Gene Expression Wash Buffer 2** needs to be warmed overnight. Make sure that you prepare the wash buffer the night before your planned microarray wash step. Do this in two steps: "Step 1. Add Triton X-102 to Gene Expression wash buffers" on page 30 and "Step 2. Prewarm Gene Expression Wash Buffer 2" on page 30 the night before you wash the microarray slides.

Microarray Wash

Step 1. Add Triton X-102 to Gene Expression wash buffers

Add the Triton X-102 to Gene Expression wash buffer 1 and 2 when the cubitainer of wash buffer is first opened.

Do this step to *both* Gene Expression wash buffer 1 and 2 before use.

1. Open the cardboard box with the cubitainer of wash buffer and carefully remove the outer and inner caps from the cubitainer.
2. Use a pipette to add 2 mL of the provided 10% Triton X-102 into the wash buffer in the cubitainer.
3. Replace the original inner and outer caps and mix the buffer carefully but thoroughly by inverting the container 5 to 6 times.
4. Carefully remove the outer and inner caps and install the spigot provided with the wash buffer.
5. Prominently label the wash buffer box to indicate that Triton X-102 has been added and indicate the date of addition.

Step 2. Prewarm Gene Expression Wash Buffer 2

The temperature of the Gene Expression Wash Buffer must be at 37°C for optimal performance.

Warm the **Gene Expression Wash Buffer 2** to 37°C as follows:

1. Dispense 1000 mL of Gene Expression Wash Buffer 2 directly into a sterile 1000-mL bottle. Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.
2. Tightly cap the 1000-mL bottle and place in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and place it in a 37°C water bath the night before washing the arrays.
3. Place slide-staining dish #3 (see Table 10 on page 32 for numbering of the wash dishes) into a 1.5 L glass dish three-fourths filled with water. Warm to 37°C by storing overnight in an incubator set to 37°C.

Step 3. Prepare the equipment

Always use designated clean equipment when doing the hybridization and wash steps. Designate and dedicate dishes to miRNA experiments.

Wash all dishes, racks, and stir bars with Milli-Q water.

1. Run copious amounts of Milli-Q water through the staining dish.
2. Empty out the water collected in the dish.
3. Repeat steps 1 and 2 at least 5 times, as it is necessary to remove any traces of contaminating material.
4. Discard the Milli-Q water.

CAUTION Some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q water.

Step 4. Wash the microarray slides

NOTE The microarray wash procedure for Agilent's miRNA platform must be done in environments where ozone levels are 50 ppb or less. For environments in which ozone levels are over 50 ppb, use the ozone-barrier slide cover.

NOTE When setting up the apparatus for the washes, be sure to do so near the water bath containing the pre-warmed Wash 2 solutions.

Table 10 lists the wash conditions for the wash procedure.

Table 10 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	GE Wash Buffer 1	Room temperature	
1st wash	2	GE Wash Buffer 1	Room temperature	5 minutes
2nd wash	3	GE Wash Buffer 2	37°C	5 minutes

1. Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.
2. Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Gene Expression Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
3. Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Gene Expression Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Gene Expression Wash Buffer 2 at 37°C. Use a thermometer to check the temperature.
4. Remove one hybridization chamber from the incubator and record the time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely. Make note of any significant loss of hybridization volume.

5 Prepare the hybridization chamber disassembly.

   a Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.

   b Slide off the clamp assembly and remove the chamber cover.

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Gene Expression Wash Buffer 1.

6 With the sandwich completely submerged in Gene Expression Wash Buffer 1, pry the sandwich open from the barcode end only:

   a Slip one of the blunt ends of the forceps between the slides.

   b Gently turn the forceps upwards or downwards to separate the slides.

   c Let the gasket slide drop to the bottom of the staining dish.

   d Remove the microarray slide and place into the slide rack in slide-staining dish #2 containing Gene Expression Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

7 Repeat steps step 4 through step 6 for up to five additional slides in the group. For uniform washing, do up to a maximum of six disassembly procedures yielding six microarray slides.

8 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4, or a moderate speed setting, for 5 minutes.

9 Transfer slide rack to slide-staining dish #3 containing Gene Expression Wash Buffer 2 at 37°C. Stir using setting 4, or a moderate speed setting, for 5 minutes.

CAUTION You must maintain Wash 2 at 37°C for the duration of the wash step. Failure to do so may result in alterations of stringency, which may reduce the consistency of experimental results.

10 Slowly remove the slide rack minimizing droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

11 Discard the used Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2.

12 Repeat step 1 through step 11 for the next group of six slides using fresh Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 pre-warmed to 37°C.

13 Put the slides in a slide holder:

Figure 2 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner B or C only:

- In environments in which the ozone level exceeds 50 ppb, immediately put the slides with Agilent barcode facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in Figure 3.

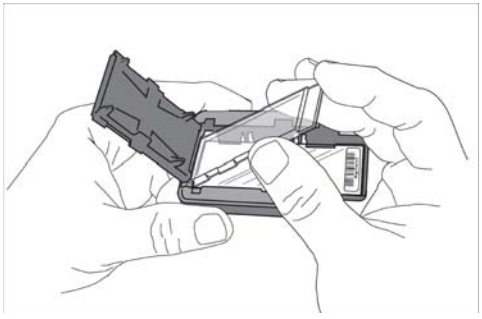

Figure 3 Inserting the ozone-barrier slide cover (for Scanner B and C)

- In environments in which the ozone level is below 50 ppb, put the slides with Agilent barcode facing up in a slide holder.

14 Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N_2 purge box, in the dark.

NOTE Use fresh Gene Expression Wash Buffer 1 and 2 for each wash group (up to 6 slides).

NOTE

The wash buffer in the disassembly dish may be pink due to the presence of unincorporated cyanine 3 dye. Discard the used wash buffer according to appropriate institutional guidelines. You can use activated charcoal to absorb the unincorporated cyanine 3 dye from the used wash buffer, if needed. Extractors from Sigma (p/n Z361569) can be used for this purpose.

Scanning and Feature Extraction

This section describes how to scan and extract data from miRNA microarrays.

Refer to the Agilent Microarray Scanner *User Guide* for more information on how to use the scanner.

Table 11 Scanner to GeneSpring Workflow version compatibility

Microarray Format	Scanner version	Scan Control version	Scan settings/profile	Spike-In	Feature Extraction version	GeneSpring version
8x60K	SureScan	9.X	AgilentG3_miRNA	Yes	10.7.3 or later	11.5 or later
8x60K	SureScan	9.X	AgilentG3_miRNA	No	10.5 or later	10.0 or later
8x15K	SureScan	9.X	AgilentHD_miRNA	Yes	10.7.3 or later	11.5 or later
8x15K	SureScan	9.X	AgilentHD_miRNA	No	10.5 or later	10.0 or later
8x60K	C	8.X	AgilentG3_miRNA	Yes	10.7.3 or later	11.0 or later
8x60K	C	8.X	AgilentG3_miRNA	No	10.5 or later	10.0 or later
8x15K	C	8.X	AgilentHD_miRNA	Yes	10.7.3 or later	11.0 or later
8x15K	C	8.X	AgilentHD_miRNA	No	10.5 or later	10.0 or later
8x15K	B	7.X	See Table 12 on page 39	Yes	10.7.3 or later	11.0 or later
8x15K	B	7.X	See Table 12 on page 39	No	9.5.3 or later	10.0 or later

Step 1. Scan the slides

Agilent Scanner Settings

NOTE Make sure the scanner has been turned on for at least 15 minutes before you continue.

1 Assemble the slides into a SureScan or Scanner C or B slide holder. Place the slides into the slide holder such that the numeric barcode side is visible (*not* the "Agilent"-labeled barcode side). Refer to "General Microarray Layout and Orientation" on page 58.

2 Place assembled slide holders into either the SureScan cassette or Scanner B or C carousel.

3 Verify scan settings for miRNA.

- If you use a SureScan or Scanner C scanner, then select the protocol or profile **AgilentHD_miRNA** for the 8x15K format, and **AgilentG3_miRNA** for the 8x60K format. All the appropriate settings are set as default and you need not change anything else. See Figure 4 for the version 8.x Scan control dialog box.

CAUTION Make sure you scan 8x60K microarrays at 3 microns. Feature Extraction cannot extract the data if G3 microarrays are scanned at 5 microns.

If neither profiles are on your local system, download them from www.agilent.com/chem/scanner, under **Download Software**. Download and unzip the profile. Within the Scan Control Software, select **Tools > Profile Editor > Import**, then browse for the location of the scan profile file, and select **Open**. Two profiles will then be added to the available list of profiles.

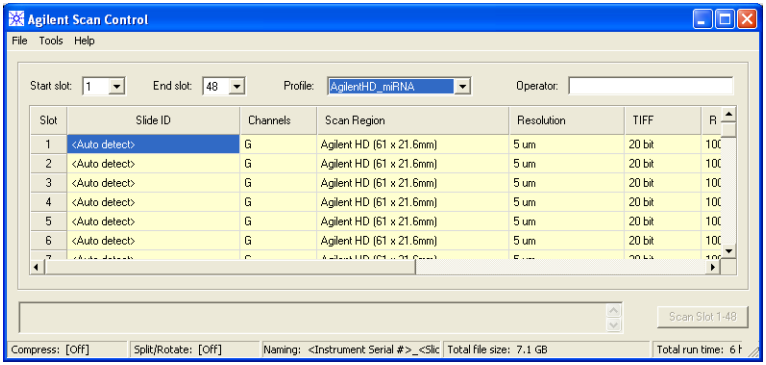

Figure 4 Scan Control dialog box with default settings for Scanner C

- If you use a B scanner with Scan control software version 7.x, you need to verify and possibly change the scan settings appropriate for miRNA scans. Figure 5 shows the version 7.x Scan Control dialog box. Table 12 shows the scan settings appropriate for miRNA scans.

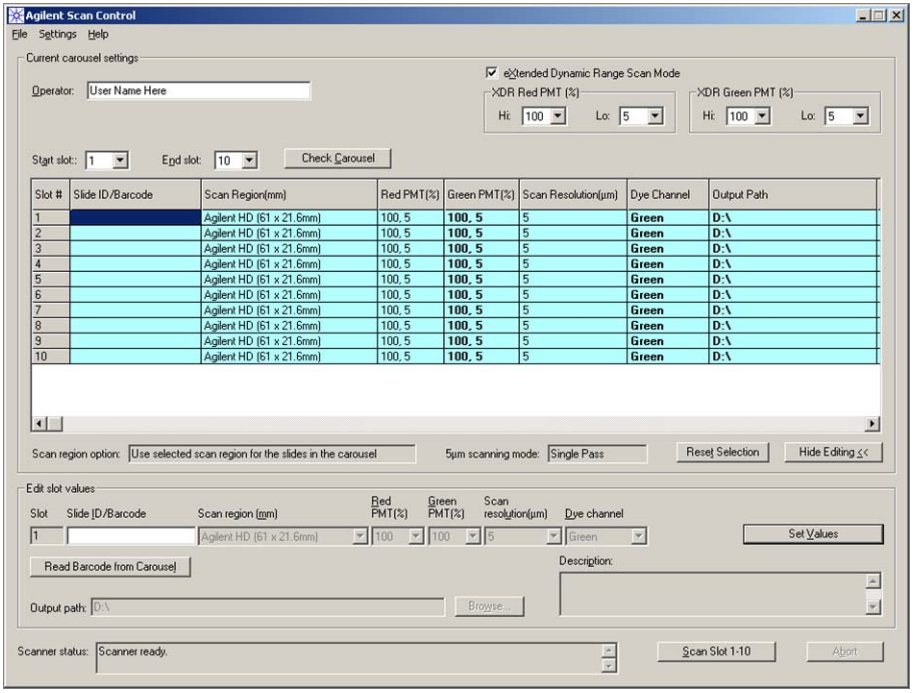
Agilent Scan Control
File Settings Help
Current carousel settings
Operator: User Name Here
eXtended Dynamic Range Scan Mode
XDR Red PMT (%)
Hi: 100 Lo: 5
XDR Green PMT (%)
Hi: 100 Lo: 5
Start slot: 1 End slot: 10 Check Carousel
Slot # | Slide ID/Barcode | Scan Region(mm) | Red PMT(%) | Green PMT(%) | Scan Resolution(µm) | Dye Channel | Output Path
1 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
2 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
3 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
4 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
5 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
6 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
7 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
8 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
9 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
10 | Agilent HD (61 x 21.6mm) | 100, 5 | 100, 5 | 5 | Green | D:\
Scan region option: Use selected scan region for the slides in the carousel
5µm scanning mode: Single Pass
Reset Selection
Hide Editing <<
Edit slot values
Slot | Slide ID/Barcode | Scan region (mm) | Red PMT(%) | Green PMT(%) | Scan resolution(µm) | Dye channel
1 | Agilent HD (61 x 21.6mm) | 100 | 100 | 5 | Green
Set Values
Read Barcode from Carousel
Description:
Output path: D:\
Browse...
Scanner status: Scanner ready.
Scan Slot 1-10
Abort

Figure 5 Scan Control dialog box with miRNA setting for Scanner B

Table 12 Scan Settings

Scan Setting	Values for 8x15K Formats
Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (µm)	5
5µm scanning mode	Single Pass
eXtended Dynamic range	(selected)
Dye channel	Green
Green PMT	XDR Hi 100% XDR Lo 5%

CAUTION The scanner settings for miRNA microarray scanning are not the same as the default XDR settings for version 7.x.

For version 7.X, to change any settings, click **Settings > Modify Default Settings**. A window pops up from which you can change the settings.

4 Select settings for the automatic file naming
   - **Prefix 1** is set to **Instrument Serial Number**
   - **Prefix 2** is set to **Array Barcode**

5 Verify that the Scanner status in the main window says **Scanner Ready**.

6 Click **Scan Slot *m*-*n*** on the Scan Control main window where the letter ***m*** represents the Start slot where the first slide is located and the letter ***n*** represents the End slot where the last slide is located.

Step 2. Extract data using Agilent Feature Extraction Software

Feature Extraction (FE) is the process by which information from probe features is extracted from microarray scan data, allowing researchers to measure miRNA expression in their experiments. To get the most recent Feature Extraction software for miRNA expression, go to the Agilent Web site at www.agilent.com/chem/fe. Refer to the Agilent Feature Extraction Software *User Guide* or *Reference Guide* for more information to use the software.

FE version 9.5.3 or higher is required for extraction of .tif images of Agilent miRNA microarrays scanned on an Agilent Scanner. FE version 10.7 gives you the option to automatically download new grid templates, protocols and QC metrics (QCM or QCMT) via eArray, and supports analysis of the spike-ins miRNA. See "Automatic Download from eArray" on page 47 for configuration. Refer to Table 11 on page 36 to determine the version of Feature Extraction you need to use.

FE version 10.7 protocol uses surrogates in determining the gTotalGeneSignal which eliminates negative expression values. Earlier versions of FE and FE protocols do not use surrogates and may create negative gTotalGeneSignal values.

1 Open the Agilent Feature Extraction (FE) program.

   To get the most recent Feature Extraction protocols for miRNA, go to the Agilent Web site at www.agilent.com/chem/feprotocols.

2 Add the images (.tif) to be extracted to the FE Project.

   a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the **Project Explorer** and select **Add Extraction...**

   b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the Shift or Ctrl key when selecting.

   The FE program automatically assigns a default grid template and protocol for each extraction set, if the following conditions are met:

   - For auto assignment of the grid template, the image must be generated from an Agilent scanner.
   - For auto assignment of the miRNA FE protocol, the **default miRNA protocol** must be specified in the FE Grid Template properties.

     To access the FE Grid Template properties, double-click on the grid template in the Grid Template Browser.

3 Set FE Project Properties.

a Select the **Project Properties** tab.

b In the **General** section, enter your name in the **Operator** text box.

c In the **Output** section, verify that at least the following default settings as shown in Figure 6 on page 43 are selected.

d For versions of FE earlier than 10.7.3, in the **Other** section, from the QC Metric Set drop-down list, select **miRNA_QCMT_Jan09** as shown in Figure 6 on page 43**.**

If the QC Metric Set is not available to select from the pull down menu, you must add it to the QC Metric Set Browser. To add, right-click inside the QC Metric Set Browser, and click **Add**. Browse for the QC Metric Set file and click **Open** to load the QC Metric Set into the FE database.

If you don't have a local copy of the QC Metric Set or would like to download the latest QC Metric Set, you can do so from Agilent Web site at www.agilent.com/chem/feqcmetrics. After downloading, you must add the QC Metric Set to the QC Metric Set Browser.

After a new QC Metric Set is added to the QC Metric Set Browser, remember to specify the default protocol for the new QC Metric Set if you want the Feature Extraction program to automatically assign a FE QC Metric Set to an extraction set.

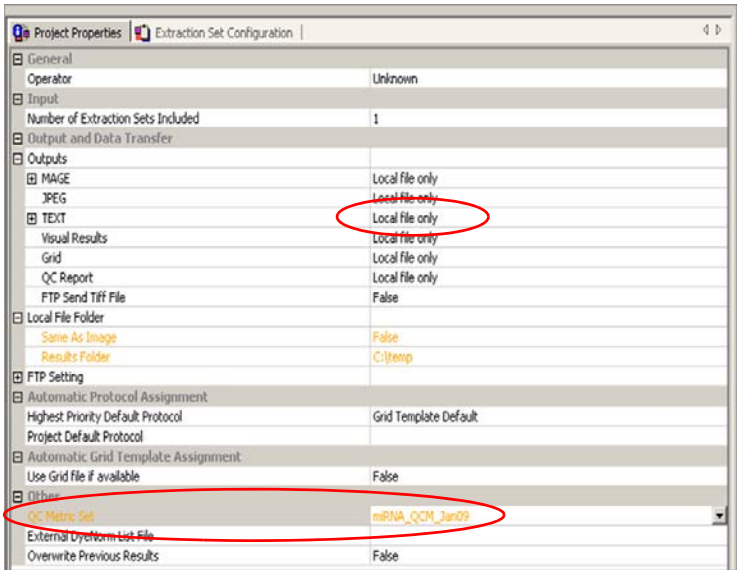

Figure 6 Feature Extraction version 9.5 dialog box

4 Check the Extraction Set Configuration.

For FE versions earlier than version 10.7.3:

a Select the **Extraction Set Configuration** tab.

b Verify that the correct grid template is assigned to each extraction set in the **Grid Name** column. To assign a different grid template to an extraction set, select one from the pull down menu.

If a grid template is not available to select from the pull down menu, you must add it to the Grid Template Browser. To add, right-click inside the Grid Template Browser, select **Add**. Browse for the design file (.xml) and click **Open** to load grid template into the FE database.

To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent Web site at www.agilent.com/chem/downloaddesignfiles. After downloading, you must add the grid templates to the Grid Template Browser.

After a new grid template is added to the Grid Template Browser, remember to specify the default protocol for the new grid template if you want the Feature Extraction program to automatically assign a FE protocol to an extraction set.

c Verify that the correct protocol is assigned to each extraction set in the **Protocol Name** column. For Agilent miRNA microarrays, select **miRNA_107_Sept09**.

The protocols automatically distinguish the formats for processing the data.

If a protocol is not available to select from the pull down menu, you must import it to the FE Protocol Browser. To import, right-click **FE Protocol Browser**, select **Import**. Browse for the FE protocol (.xml) and click **Open** to load the protocol into the FE database. Visit the Agilent Web site at www.agilent.com/chem/feprotocols to download the latest protocols.

NOTE When the Agilent XDR scanned images are added to Feature Extraction software version 9.5 or later, the High and Low images are automatically combined for data extraction.

NOTE 20-bit single images from the C Scanner are equivalent to 16-bit XDR images from the B Scanner.

5 Save the FE Project (.fep) by selecting **File > Save As** and browse for desired location.

6 Verify that the icons for the image files in the FE Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected. If needed, reselect the extraction protocol for that image file.

7 Select **Project > Start Extracting**.

8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting Spot Finding at the Four Corners of the Array. See Figure 7.

If a QC Metric Set has been assigned to the FE Project, you can view the results of the metric evaluation in three ways:

- Project Run Summary - includes a summary sentence.
- QC Report - includes both a summary on the header and a table of metric values. Figure 7 shows a QC report for an miRNA microarray with the use of spike-in miRNAs.
- QC Chart - includes a view of the values of each metric compared across all extractions in FE Project.

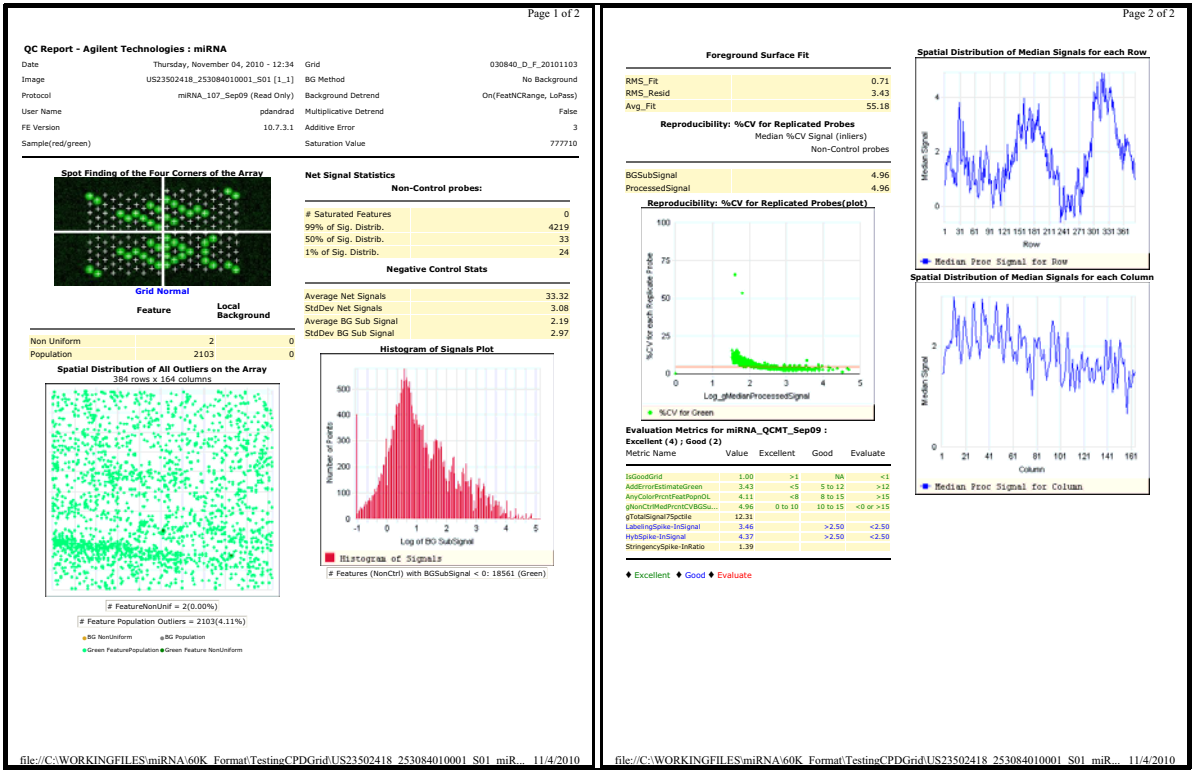
Page 1 of 2
QC Report - Agilent Technologies : miRNA
Spot Finding of the Four Corners of the Array
Grid Normal
Feature
Local Background
Non Uniform 2 0
Population 2103 0
Spatial Distribution of All Outliers on the Array
384 rows x 164 columns
FeatureNonUnif = 2(0.00%)
Feature Population Outliers = 2103(4.11%)
Net Signal Statistics
Non-Control probes:
Saturated Features 0
99% of Sig. Distrib. 4219
50% of Sig. Distrib. 33
1% of Sig. Distrib. 24
Negative Control Stats
Average Net Signals 33.32
StdDev Net Signals 3.08
Average BG Sub Signal 2.19
StdDev BG Sub Signal 2.97
Histogram of Signals Plot
Number of Feats
Log of BG SubSignal
Histogram of Signals
Features (NonCtrl) with BGSubSignal < 0: 18561 (Green)
file://C:\WORKINGFILES\miRNA\60K_Format\TestingCPDGrid\US23502418_253084010001_S01_miR... 11/4/2010
Page 2 of 2
Foreground Surface Fit
RMS_Fit 0.71
RMS_Resid 3.43
Avg_Fit 55.18
Reproducibility: %CV for Replicated Probes
Median %CV Signal (inliers)
Non-Control probes
BGSubSignal 4.96
ProcessedSignal 4.96
Reproducibility: %CV for Replicated Probes(plot)
%CV for each Replicate Probe
Log_gMedianProcessedSignal
%CV for Green
Evaluation Metrics for miRNA_QCMT_Sep09 :
Excellent (4) ; Good (2)
Metric Name Value Excellent Good Evaluate
IsGoodGrid 1.00 >1 NA <1
AddErrorEstimateGreen 3.43 <5 5 to 12 >12
AnyColorPrcntFeatPopnOL 4.11 <8 8 to 15 >15
gNonCtrlMedPrcntCVBGSu... 4.96 0 to 10 10 to 15 <0 or >15
gTotalSignal75pctile 12.31
LabelingSpike-InSignal 3.46 >2.50 <2.50
HybSpike-InSignal 4.37 >2.50 <2.50
StringencySpike-InRatio 1.39
♦ Excellent ♦ Good ♦ Evaluate
Spatial Distribution of Median Signals for each Row
Median Signal
Row
Median Proc Signal for Row
Spatial Distribution of Median Signals for each Column
Median Signal
Column
Median Proc Signal for Column
file://C:\WORKINGFILES\miRNA\60K_Format\TestingCPDGrid\US23502418_253084010001_S01_miR... 11/4/2010

Figure 7 Example of a QC Report for 8x15K miRNA microarray, generated by Feature Extraction version 10.7.3 with the use of Spike-In miRNAs.

Agilent Feature Extraction Result Files

Agilent Feature Extraction software generates two text files for each miRNA microarray. The text file that uses the name *ScannerNum_BarcodeNum*_S0#_*FEProtocol_Array_Num*.txt (for example, "US22502705_251911810634_S01_miRNA-v1_95_May07_1_1.txt") includes all feature, probe and gene level data, as well as array level statistics and parameters. Use these files for analysis and to review the QC metrics in the GeneSpring GX version 10 or later.

The other text file, the "GeneView" file, uses the name *BarcodeNum*_SO#_*Array_Num*_GeneView.txt (for example, "251911810634_S01_1_1_GeneView.txt"). The GeneView file provides the summarized TotalGeneSignal, TotalGeneError, and a detection flag for each miRNA gene. This simple file is appropriate for any analyses and can be loaded into GeneSpring GX version 9.0, or similar software for analysis. However, it does not contain the QC metric data for review. The "gTotalGeneSignal" column has been background subtracted and outlier rejected and is appropriate for use as the measured intensity for each miRNA gene without further manipulation.

Please refer to the Agilent Feature Extraction Software *User Guide* or *Reference Guide* for more detail on the data summarization algorithms and for information on other columns in the feature extraction result files.

Automatic Download from eArray

Feature Extraction version 10.7 or later can automatically download Grid Templates, protocols and QC metrics (QCM or QCMT). To set this up, in the eArray Login Setting dialog box, under **Advanced Options**, click **Use eArray server during extraction**. See Figure 8.

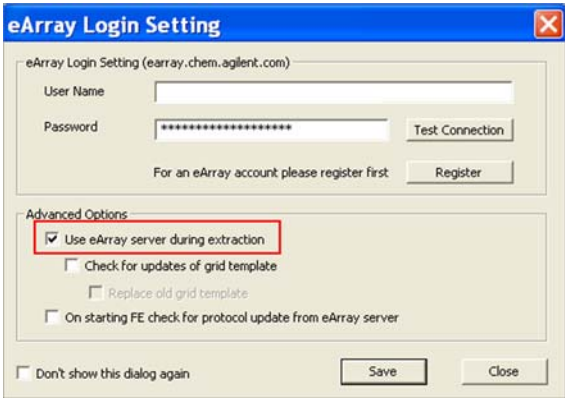

Figure 8 eArray Login Setting

3 Characterization of Total RNA

Step 1. Prepare for characterization of total RNA 51
Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer 52

Although this step is optional in the miRNA microarray work-flow, Agilent strongly recommends that you characterize the total RNA for sample integrity and possible presence of contaminants. This chapter gives a general guideline for total RNA and small RNA characterization prior to proceeding with labeling and hybridization.

Depending on the method used for purification of the total RNA, your sample may have contaminants such as phenol, salts, and/or genomic DNA. You can check the absorption spectrum from 220 nm to about 300 nm to detect some of these possible contaminants. In the absorption spectra, you should see only one peak with an absorption maximum at 260 nm. You may also see a shoulder of an additional peak at < 220 nm that overlaps with and inflates the absorption at 260 nm. The level of inflation can be seen on the absorption spectrum. If the absorption spectrum is not available, calculate the ratio of absorbances at 260/230 nm. This ratio should be greater than 1.8. If the ratio is less than 1.8, then you need to purify the sample further by doing additional extractions with chloroform, followed by an ethanol precipitation. To ensure recovery of the RNA, do this purification with a large (microgram) quantity of total RNA.

Unfortunately, absorption spectra cannot differentiate between RNA and DNA. Contamination with DNA can result in overestimation of total RNA amounts, and may lead to decreased sensitivity on the miRNA assay due to the effective decrease in the amount of total RNA added.

Characterization of the input total RNA using the Agilent 2100 bioanalyzer provides information on the sample quality prior to labeling and hybridization. You can use the RNA 6000 Nano kit to analyze total RNA with the appropriate assay at the assay specified concentration. For low concentration samples, use the RNA 6000 Pico kit.

The small RNA assay characterizes the total RNA sample with an emphasis on the small RNA content of which a fraction is miRNA. This can be done using the Small RNA Kit along with the Small RNA assay on the Agilent 2100 bioanalyzer.

For assessment of total RNA quality, the Agilent 2100 Expert Software automatically provides a RNA Integrity Number (RIN). RIN provides a quantitative value for RNA integrity that facilitates the standardization of quality interpretation. Users should define a minimum threshold RIN number based on correlative data in order to eliminate experimental bias due to poor RNA quality.

For general assistance on the evaluation of total RNA with emphasis on the RNA integrity number, refer to the application note “RNA integrity number (RIN) - Standardization of RNA quality control,” part number 5989-1165EN.

To download application notes and for more information regarding Agilent’s 2100 bioanalyzer along with the various kits offered for analysis of both total RNA and small RNA, visit the Web site at www.agilent.com/chem/labonachip.

Step 1. Prepare for characterization of total RNA

- Refer to Table 13 and Table 14 to make sure that you have the appropriate analyzer, kits, and compatible assays.

Table 13 Analyzer and Kits

Description	Company and part no.
2100 Bioanalyzer	Agilent p/n G2938C or G2939A
RNA 6000 Nano Kit	Agilent p/n 5067-1511
RNA 6000 Pico Kit	Agilent p/n 5067-1513
Small RNA Kit	Agilent p/n 5067-1548

Table 14 Recommended Assays

Description	Compatible Assay
RNA 6000 Nano Kit	Agilent Eukaryote Total RNA Nano Assay Qualitative range 5 to 500 ng/µL
RNA 6000 Pico Kit	Agilent Eukaryote Total RNA Pico Assay Qualitative range 50 to 5000 pg/µL in water
Small RNA Kit	Agilent Small RNA Assay • 50 to 200 pg miRNA • 1 to 10 ng/µL of purified small RNA < 200 nt • 10 to 50 ng/µL of total RNA

Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer

To measure the total RNA integrity, purity and concentration, use one of the Agilent RNA 6000 kits. Follow the instructions in the kit.

To measure the small RNA amount and profile, use the Agilent Small RNA kit. Follow the instructions in the kit.

1. Ensure the 2100 bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 expert software (version B.02.04 or higher), switch on the 2100 bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

RNA 6000 - Total RNA

Total RNA characterization provides information on the quality of the total RNA, which may impact the overall RNA quantification and the microarray miRNA profile.

The resulting electropherogram typically has at least two distinct peaks representing the 18S and 28S ribosomal RNA. The peak representing the lower marker can also be seen as shown in Figure 9. The presence of the 5S RNA peak, as shown in Figure 9, depends on the purification method, and generally shows lower abundance in column purified total RNA. Figure 9 demonstrates the differences in the peaks with varying degrees of total RNA integrity and provides RNA Integrity Number (RINs) as examples. Make sure you define a minimum threshold RIN number based on correlative data in order to eliminate experimental bias due to poor total RNA quality.

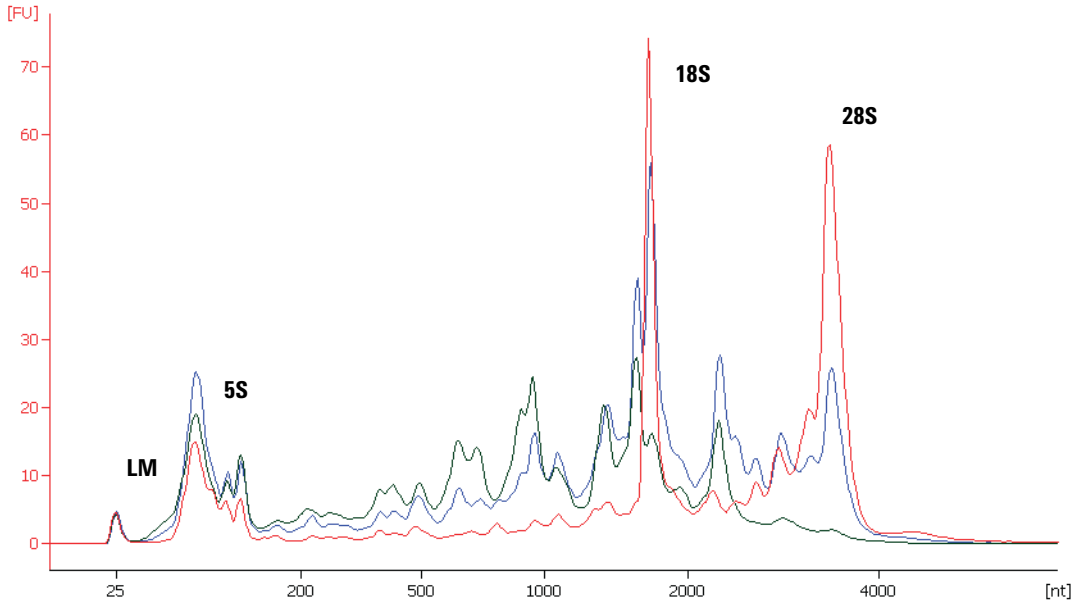

Figure 9 Analysis of (human) total RNA with the Eukaryote total RNA Nano assay using three different samples with decreasing integrity: Red, RIN 8.4; Blue, RIN 5.9; Green, RIN 3,6. Characteristic regions for ribosomal peaks and the lower marker (LM) are displayed.

Small RNA - miRNA in total RNA

The small RNA assay characterizes the total RNA sample with an emphasis on the small RNA content of which a fraction is miRNA.

The resulting electropherogram typically has a number of bands or peaks representing small RNAs ranging in size from 10 to 150 nt. The miRNA portion is represented by the band or peak ranging in size from 10 to 40 nt. These bands or peaks will vary in abundance depending on the total miRNA preparation. Figure 10 shows an example of the resulting electropherogram of the small RNA assay for a total RNA sample.

You can record the results of the small RNA assay to correlate to your miRNA microarray data. However, the small RNA assay results may not necessarily be an indicator for the miRNA content in the sample, and the miRNA microarray assay is a more sensitive technique for detecting low levels of miRNAs.

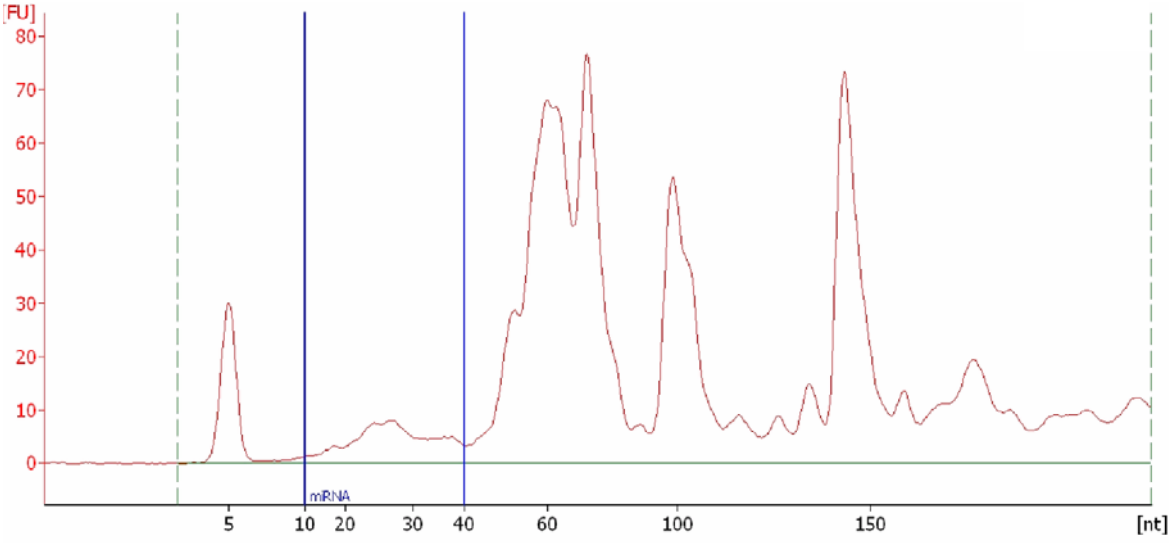

Figure 10 Characterization of small RNA within a total RNA sample. miRNA is typically found in the 10 to 40 nt region, which is a subset of the small RNA that range from 10 to 150 nt

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This chapter contains reference information related to the protocol, and the limited use license.

Supplemental User Guides

First-time users of Agilent's oligo microarray system, please refer to the following user guides for detailed descriptions and operation recommendations for each of the hardware and software components used in the miRNA platform workflow. The user guides were included with your Agilent hardware and software. You can also download them from the Agilent web site at www.agilent.com/chem/dnamanuals-protocols. (Search on publication type "Manuals".)

- G2534A Microarray Hybridization Chamber User Guide
- G2545A Hybridization Oven User Manual
- G2565CA or G2565BA Microarray Scanner System User Guide *or* the G4900DA SureScan Microarray Scanner User Guide
- Feature Extraction Installation Guide
- Feature Extraction Quick Start Guide
- Feature Extraction User Guide
- Feature Extraction Reference Guide

Microarray Handling Tips

Each microarray is printed on the side of the glass slide containing the "Agilent"-labeled barcode. This side is called the "active" side. The numeric barcode is on the inactive side of the slide.

CAUTION You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides.

In this "processing and hybridization" procedure, the hybridization mixture is applied directly to the gasket slide, and not to the active side of the oligo microarray. Instead, the active side of the oligo microarray is placed on top of the gasket slide to form a "sandwich slide" pair.

To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

Never allow the microarray surface to dry out during the hybridization process and washing steps.

Do not write anywhere on the array or gasket slide, as you can introduce severe artifacts on the scanned images.

General Microarray Layout and Orientation

Agilent oligo microarray (8 microarray/slide format) as imaged on Agilent microarray scanners

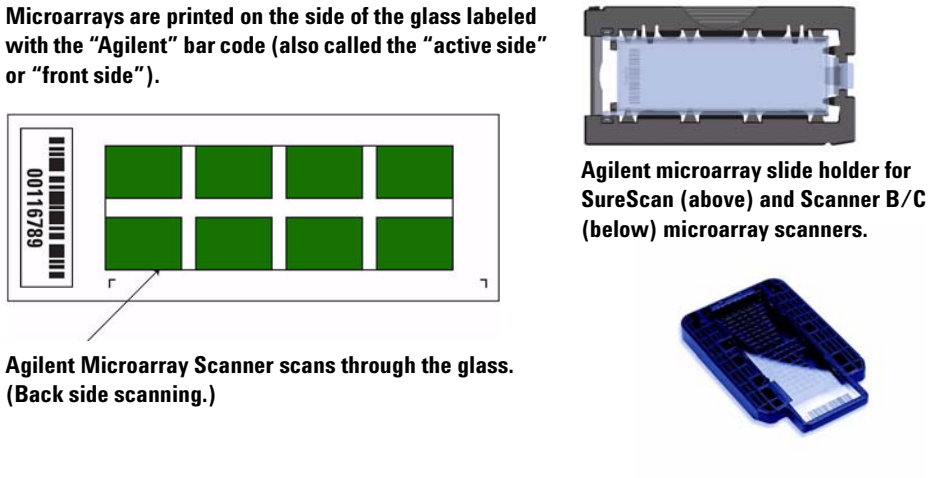
Microarrays are printed on the side of the glass labeled with the "Agilent" bar code (also called the "active side" or "front side").
0011678
Agilent Microarray Scanner scans through the glass. (Back side scanning.)
Agilent microarray slide holder for SureScan (above) and Scanner B/C (below) microarray scanners.

Figure 11 Agilent microarray slide and slide holder

Agilent oligo microarray formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch × 3-inch glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent microarray slide holder with the "Agilent"-labeled barcode facing down. In this orientation, the "active side" containing the microarray is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, non-active side of the slide, is visible.

Figure 11 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays.

Array/Sample tracking on an 8-pack array slide

Use the form below to make notes to track your samples on a 8-pack array slide.

Position the gasket slide in the SureHyb chamber base with the label to the left and load the samples: top row, left to right, then lower row, left to right. The array suffix assignments from Feature Extraction will then occur in the order shown.

Arrays

	Array 1_1	**Array 1_2**	**Array 1_3**	**Array 1_4**
BARCODE	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Sample: _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____	**Sample:** _____ _____ _____
	Array 2_1	**Array 2_2**	**Array 2_3**	**Array 2_4**

Barcode Number _____

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In This Book

This document describes Agilent's recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent oligonucleotide microarrays for microarray-based miRNA systems.

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Version 2.4, August 2011
Revision A3

G4170-90011

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